

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/31028 A3

- (51) International Patent Classification⁷: C12N 15/53, 9/02, C12Q 1/66
- (21) International Application Number: PCT/GB00/04133
- (22) International Filing Date: 26 October 2000 (26.10.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
9925161.3 26 October 1999 (26.10.1999) GB
0016744.5 10 July 2000 (10.07.2000) GB
- (71) Applicant (for all designated States except US): **THE SECRETARY OF STATE FOR DEFENCE** [GB/GB]; DSTL, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **SQUIRRELL, David, James** [GB/GB]; DSTL, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). **MURPHY, Melenie, Jane** [GB/GB]; TSTL, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). **PRICE, Rachel, Louise** [GB/GB]; DSTL, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). **WHITE, Peter, John** [GB/GB]; DSTL, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). **WILLEY, Tara, Louise** [GB/GB]; The Babraham Institute, Babraham Hall, Village of Babraham, Cambridge CB2 4AT (GB).
- (74) Agent: **SKELTON, Stephen, Richard**; D/IPR, Formalities Section, Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
- (88) Date of publication of the international search report:
2 May 2002
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/31028 A3

(54) Title: **MUTANT LUCIFERASE**

(57) Abstract: A recombinant protein having luciferase activity and at least 60 % similarity to a wild-type luciferase wherein in the sequence of the enzyme, the amino acid residue corresponding to residue 357 in *Photinus pyralis* luciferase is mutated as compared to the corresponding wild-type luciferase, such that the luciferase enzyme is able to emit light at a different wavelength as compared to the corresponding wild-type luciferase and/or has enhanced thermostability as compared to the corresponding wild-type luciferase. In general, the residue corresponding to 357 in *Photinus pyralis* luciferase is changed from an acidic amino acid to a non-acidic amino acid and preferably an uncharged polar amino acid such as tyrosine. Mutant luciferases in accordance with the invention can produce a large (50nm) wavelength shift in emitted light and have good thermostability. The resultant colour shift can be reversed by addition of coenzyme A. These properties make the mutant particularly useful in a variety of assays.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 00/04133

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N9/02 C12Q1/66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 14336 A (PROMEGA CORPORATION (US); WOOD KEITH V.; HALL MARY P.) 25 March 1999 (1999-03-25) page 5, line 1-15; figure 19 page 36 -page 40; example 4 Mutant Luc78-0B10 page 67 Mutant Luc90-185 page 69 page 71 -page 72; tables 2,3 page 77 -page 106; claims figures 36,38,39,41,43 --- -/--	1,2,5,6, 11,12, 14-16, 18-23

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

20 July 2001

Date of mailing of the international search report

01/08/2001

Name and mailing address of the ISA

European Patent Office, P.O. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/GB 00/04133

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	<p>WO 01 20002 A (PROMEGA CORPORATION (US); WOOD KEITH V.; HALL MARY P.; GRUBER MONIKA) 22 March 2001 (2001-03-22)</p> <p>Mutant Luc78-0B10, SEQ ID NO:6,19; Mutant Luc90-1B5, SEQ ID NO:11,24; Mutant Luc133-1B2, SEQ ID NO:42,44; Mutant Luc146-1H2, SEQ ID NO:43,45 page 8 -page 12; figures 19A,32,36,42,43,55,56,57,58 page 27 -page 32 page 52 -page 81</p> <p>---</p>	<p>1,2,5,6, 11,12, 14-16, 18-23</p>
A	<p>LI YE ET AL.: "Cloning and sequencing of a cDNA for firefly luciferase from Photuris pennsylvanica" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1339, 25 April 1997 (1997-04-25), pages 39-52, XP000909154 ISSN: 0006-3002 cited in the application page 40; table 1 page 42 -page 44; figure 2</p> <p>---</p>	
A	<p>WO 95 25798 A (SECRETARY STATE DEFENCE UNITED KINGDOM; LOWE; WHITE; MURRAY; SQUIRREL) 28 September 1995 (1995-09-28) cited in the application</p> <p>---</p>	
A	<p>WHITE P.J. ET AL: "Improved thermostability of the North American firefly luciferase: saturation mutagenesis at position 354" BIOCHEMICAL JOURNAL, vol. 319, 1996, pages 343-350, XP002097112 ISSN: 0264-6021</p> <p>---</p>	
A	<p>VIVIANI V.R. ET AL.: "Cloning, sequence analysis and expression of active Phrixothrix railroad-worms luciferases: relationship between bioluminescence spectra and primary structures" BIOCHEMISTRY, vol. 38, no. 26, 29 June 1999 (1999-06-29), pages 8271-8279, XP002172177 cited in the application page 8275; figure 5</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat'l Application No

PCT/GB 00/04133

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9914336	A	25-03-1999	AU	9492198 A	05-04-1999
			EP	1015601 A	05-07-2000
WO 0120002	A	22-03-2001	AU	2215600 A	17-04-2001
WO 9525798	A	28-09-1995	AU	697883 B	22-10-1998
			AU	1954595 A	09-10-1995
			BR	9507140 A	30-09-1997
			CA	2186144 A	28-09-1995
			CN	1149318 A	07-05-1997
			EP	0751996 A	08-01-1997
			FI	963741 A	20-11-1996
			GB	2301592 A,B	11-12-1996
			JP	9510610 T	28-10-1997
			NO	963983 A	23-09-1996
			US	6132983 A	17-10-2000

PATENT COOPERATION TREATY

PCT

REC'D 30 MAY 2002

WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P1247/WOD		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB00/04133	International filing date (day/month/year) 26/10/2000	Priority date (day/month/year) 26/10/1999	
International Patent Classification (IPC) or national classification and IPC C12N15/53			
Applicant THE SECRETARY OF STATE FOR DEFENCE et al.			

1. This International preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 11 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 15 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 14/05/2001	Date of completion of this report 30.05.2002
Name and mailing address of the international preliminary examining authority:  European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Macchia, G  Telephone No. +31 70 340 4078

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB00/04133

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1,2,6-8,10,13, as originally filed
18-43

3-5,9,11,12, as received on 24/04/2002 with letter of 18/04/2002
14-17

Claims, No.:

1-25 as received on 24/04/2002 with letter of 18/04/2002

Drawings, sheets:

1/24-24/24 as originally filed

Sequence listing part of the description, pages:

1-8, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
☒ filed together with the international application in computer readable form.
☐ furnished subsequently to this Authority in written form.
☐ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB00/04133

- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3, 4, 7-10, 11(b,c,d,f,g,h,i,j,k), 13, 17, 24, 25
	No:	Claims	1, 2, 5, 6, 11(a,e), 12, 14-16, 18-23
Inventive step (IS)	Yes:	Claims	7-9, 11(b,c,d,f,g,h,i,j,k), 13, 24, 25
	No:	Claims	1-6, 10, 11(a,e), 12, 14-23
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

2. Citations and explanations
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/04133

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

- D1: WO 99 14336 A (PROMEGA CORPORATION (US); WOOD KEITH V.; HALL MARY P.) 25 March 1999;
- D2: LI YE et al.: ' Cloning and sequencing of a cDNA for firefly luciferase from *Photuris pennsylvanica* ' BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1339, 25 April 1997, pages 39-52, XP000909154;
- D3: VIVIANI V.R. et al.: ' Cloning, sequence analysis and expression of active *Phrixothrix* railroad-worms luciferases: relationship between bioluminescence spectra and primary structures ' BIOCHEMISTRY, vol. 38, no. 26, 29 June 1999, pages 8271-8279, XP002172177.

The following documents D4 and D5 were not cited in the International Search Report. Copies of the documents are appended hereto:

- D4: ALBERTS B. et al.: ' MOLECULAR BIOLOGY OF THE CELL ' third edition, 1997, Garland Publishing, Inc., New York & London, pages 56 and 57;
- D5: WATSON J.D. et al.: ' MOLECULAR BIOLOGY OF THE GENE ' fourth edition, 1991, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California, page 43.
- 1.1). Document D1 relates to *Photuris pennsylvanica* luciferase mutants with increased thermostability. Among the mutants on which document D1 puts more emphasis, the mutants named " Luc78-0B10 " and " Luc90-1B5 " are indicated (D1: pages 4 and 13; tables 1 and 2). The amino acid sequences of these mutants are disclosed in D1 (D1: page 4 and figures 36 and 43) and claimed (D1: claim 21(f) and 21(k)). The amino acid sequence of the corresponding wild-type enzyme is also disclosed (D1: page 3 and figure 45). From the sequences disclosed as

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/04133

indicated above, it can be seen that, in the wild-type amino acid sequence of *Photuris pennsylvanica* luciferase, the amino acid residue corresponding to residue 357 in *Photinus pyralis* luciferase is a Valine residue. This correspondence is indicated in the sequence alignment of figure 2 of document D2, to which present application refers for the identification of residues corresponding to a certain position in the *Photinus pyralis* luciferase amino acid sequence.

The amino acid sequence alignment among the sequences of *Photinus pyralis*, *Photuris pennsylvanica* wild-type and of the mutants " Luc 78-0B10 " and " Luc 90-1B5 " luciferases, in the stretch of amino acid residues around residue 357 of *Photinus pyralis* luciferase, is indicated below. From this sequence alignment, it can be seen that the wild-type Valine residue of *Photuris pennsylvanica* luciferase, which corresponds to residue 357 in *Photinus pyralis* luciferase, is substituted in the mutants " Luc78-0B10 " and " Luc90-1B5 " with an Alanine (D1: figures 36 and 43). In this respect, it should be noted that the presence of two undefined residues, indicated by " X ", immediately before said Alanine residue, does not lead to any uncertainty with regard to the identification of the residues in the mutant luciferases which correspond to a certain residue in the wild-type sequence. In fact, as shown in the alignment below, the overall homology in the regions flanking these two undefined amino acid residues leads to no doubts about the correct alignment among the sequences, moreover, it should also be noted that there is no need of introducing gaps, in order to optimize the alignment in said region.

357

<i>P. pyralis</i>	RQGYGLTETTSAILITPEGDDKPGAVGKVVPFFFEAKVVDLDTGKTLGVNQRGEL
<i>P. penn. wt</i>	RQGYGLTETTSAVLITPDTDVRPGSTGKIVPFHAVKVVDPTTGKILGPNETGEL
78-0B10	RQGYGLTETTSAVLITPKXXARPGSTGKIVPFHAVKVVDPTTGKILGPNEPGEL
90-1B5	RQGYGLTETTSAVLITPKXXAKPGSTGKIVPFHAVKVVDPTTGKILGPNEPGEL

In addition to the previous remarks, in the amino acid sequences of mutants " Luc78-0B10 " and " Luc90-1B5 ", the following additional mutations can be

observed:

- a) a Lysine residue is present in the position corresponding to the Aspartic Acid residue in the stretch LITPDTDVR of wild-type *Photuris pennsylvanica* luciferase. This Aspartic Acid residue is the one corresponding to position E354 in *Photinus pyralis* luciferase, as indicated in the sequence alignment of D2 already mentioned (D2: figure 2) and indicated in the sequence alignment above by a dot;
- b) the Phenylalanine residue in the stretch LMAFFAKSA of wild-type *Photuris pennsylvanica* luciferase is substituted with a Leucine. This Phenylalanine residue is the one corresponding to position 295 in *Photinus pyralis* luciferase.

In addition to this, document D1 discloses and claims the nucleic acid sequences coding for said mutants " Luc78-0B10 " and " Luc90-1B5 " (D1: figures 32 and 42, claims 16(f) and 16(k), respectively). A *Bam*HI restriction site (GGATCC) is present at the beginning of both sequences. This restriction site is not present in the nucleic acid sequence encoding the luciferase, as originally isolated from *Photuris pennsylvanica* (as indicated in figure 1 of D2). Therefore, the nucleic acid sequences disclosed in figures 32 and 42 of D1 are embraced by the scope of claim 15.

An expression vector comprising said nucleic acid, *Escherichia coli* cells transformed with said vector, a method of producing said luciferase mutants, which method comprises culturing said *Escherichia coli* cell, are described in D1 (D1: pages 2-3). This description is considered to be an enabling disclosure for the subject-matter of claims 18-20 because at the priority date of present application, expression vectors, host cells and corresponding methods for the production of recombinant proteins were tools very well known to the person skilled in the art and currently used in the technical field.

The use of said luciferase mutants in a bioluminescent assay and a kit comprising said mutants and luciferin are also described in D1 (D1: pages 16-20).

- 1.2). The subject-matter of claims 1, 2 (insofar as the subject-matter of claim 2 refers to a mutant *Photuris pennsylvanica* luciferase), 5, 6, 11(a,e), 12, 14-16 and 18-23 is therefore not novel (Article 33(2) PCT).
- 2). Claims 2 (insofar as claim 2 refers to a mutant luciferase whose corresponding

wild-type sequence is from organisms other than *Photuris pennsylvanica*), 3, 4, 7-10, 11(b,c,d,f,g,h,i,j,k), 13, 17, 24 and 25 meet the requirements of Article 33(2) PCT because the subject-matter concerned in these claims was not described in the available prior art (documents D1-D3).

- 3.1). Document D1, which is considered to represent the most relevant state of the art, discloses *Photuris pennsylvanica* luciferase mutants with increased thermostability, as already commented under previous point 1.1). The subject-matter of claims 2 (insofar as claim 2 refers to a mutant luciferase whose corresponding wild-type sequence is from organisms other than *Photuris pennsylvanica*), 3, 4, 10 and 17 differs in that mutants of luciferases from organisms other than *Photuris pennsylvanica* are concerned.
- 3.2). The problem to be solved by the present invention may therefore be regarded as the provision of further luciferases with increased thermostability and/or able to emit light at a different wavelength, as compared to the corresponding wild-type luciferase.
- 3.3). The solution proposed in claims 2 (insofar as claim 2 refers to a mutant luciferase whose corresponding wild-type sequence is from organisms other than *Photuris pennsylvanica*), 3, 4, 10 and 17 (insofar as claim 17 refers to a nucleic acid comprising a sequence having at least 90% similarity to the stretch of nucleotides 9-1661 of SEQ ID NO:1) of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons: document D1 recites on pages 6-7 that the overall three-dimensional structure of all beetle luciferases is quite similar and that high thermostability can be achieved for other beetle luciferases by methods similar to the one disclosed in D1. Moreover, on page 8 of D1 it is stated that, since all beetle luciferases belong to the same structural class, they also share in the same pool of potentially stabilizing mutations. In addition to this, on page 9 of D1 it is stated that " similar results were achieved using another beetle luciferase from *Pyrophorus plagiophthalmus* ". These statements can be considered as a suggestion that the same mutations found to lead to increased thermostability in *Photuris pennsylvanica*, as disclosed in D1, may also be applied to luciferases from related organisms, as the ones disclosed in figure 17 of D1 and whose amino acid sequence is aligned in figure

19 of D1. Therefore, the disclosure of document D1 would be considered by the person skilled in the art as an incentive to mutate other beetle luciferases, in the positions corresponding to the ones mutated in the thermostable mutants " Luc78-0B10 " and " Luc90-1B5 " and using amino acid residues of the same class of the ones used in said " Luc78-0B10 " and " Luc90-1B5 " mutants.

Consequently, starting from the description of D1, a person skilled in the art would have arrived at the obtainment of mutant luciferases which fall within the scope of claims 2 (insofar as claim 2 refers to a mutant luciferase whose corresponding wild-type sequence is from organisms other than *Photuris pennsylvanica*), 3, 4, 10 and 17 (insofar as claim 17 refers to a nucleic acid comprising a sequence having at least 90% similarity to the stretch of nucleotides 9-1661 of SEQ ID NO:1) with a reasonable expectation of success and without using his inventive skill, requiring nothing extraordinary all being a matter of technical convenience.

- 4). Claims 7-9, 11(b,c,d,f,g,h,i,j,k), 13 and 17 (insofar as claim 17 refers to a nucleic acid comprising nucleotides 9-1661 of SEQ ID NO:1) meet the requirements of Article 33(3) PCT because document D1 provides no suggestion that can be considered by the skilled man as an incentive to test mutations at positions other than the ones mutated in the luciferases " Luc78-0B10 " and " Luc90-1B5 ", or mutated at the same positions of " Luc78-0B10 " and " Luc90-1B5 " but with amino acid residues belonging to a different class, as compared to the ones used in said mutants.

Claims 24 and 25 relate to a method involving the mutant luciferases mentioned above, and as such also meet the requirements of the PCT with respect to inventive step (Article 33(3) PCT).

- 5). The industrial applicability of the subject-matter of claims 1-25 is acknowledged (Article 33(4) PCT).

The following remarks are done with respect to Article 6, Rule 6 PCT.

- 6.1). Present application refers to luciferases having a mutation, as compared to the corresponding wild-type enzyme, at a position corresponding to residue 357 in *Photinus pyralis* luciferase. A definition of a residue in a luciferase which is " corresponding " to residue 357 present in the luciferase from *Photinus pyralis* is

given on page 4 of present application, reciting that corresponding regions among the enzyme sequences are readily determinable by examination of the sequences to detect the most similar regions, if necessary also by using commercially available software (*i.e.* Bestfit). Alternatively or additionally, corresponding residues can be determined by reference to the sequence alignment shown in figure 2 of document D2, already mentioned.

In this respect, the IPEA is of the opinion that this definition does not always allow to define unambiguously a residue in a luciferase, which corresponds to residue 357 of *Photinus pyralis* luciferase for the following reasons:

- i). in case the primary sequence homology was the criterium for defining residues corresponding to the one in the luciferase of reference, it should be noted that different algorithms exist for amino acid sequence alignments. Moreover, each algorithm allows the possibility of modifying the parameters used for sequence alignment. The use of said different algorithms, and/or the application of said different parameters might result in different sequence alignments, therefore making it unclear the identification of a residue corresponding to another one in a sequence of reference.

In addition to this, it should be noted that sequence alignment programs make use of the introduction of gaps, in order to optimize the alignment of the sequences to be compared. These gaps have the effect of making it uncertain the identification of "corresponding residues" falling within said gaps, or even close to it, because it might happen that a gap can be slightly shifted without affecting the efficiency of the alignment.

As a matter of fact, in figure 2 of document D2, the amino acid residues D357 of *Photinus pyralis* is aligned with the amino acid residue "E", indicated in bold in the stretch "AEGEFKL" in the luciferases from *Photuris pennsylvanica* indicated as *Ppe*(J19) and *Ppe*1(KW), due to introduction of gaps aimed at the optimization of the alignment of the whole series of sequences. If however, only the two sequences from *Photinus pyralis* and *Photuris pennsylvanica* *Ppe*(J19) or *Ppe*1(KW) luciferases were aligned, the amino acid residue in *Ppe*(J19) or *Ppe*1(KW), corresponding to D357 of *Photinus pyralis* luciferase would then be the residue "F" indicated in bold in the same stretch "AEGEFKL".

- ii). Moreover, it should be noted that the subject-matter of claim 1 embraces

luciferase mutants from any possible luciferase, even from the ones not so related to the *Photinus pyralis* luciferase of reference and, in addition to this, the claim is not limited to mutants which are mutated at one specific position, but also luciferases having at least 60% similarity to said wild-type luciferase, this adding more complexity to the possibility of finding unambiguous correspondence among amino acid residues.

- iii). In addition to this, it should be noted that in claim 1, the following functional statement: " ability to emit light at a different wavelength and/or possession of enhanced thermostability, as compared to the corresponding wild-type luciferase " does not enable the skilled person to determine which technical features are necessary to perform the stated functions (see also PCT Preliminary Examination Guidelines C-III, 4.5) and in this respect, it should be remarked that a mutation in a position which corresponds to position 357 of *Photinus pyralis* luciferase, does not always lead to a luciferase having these desired properties. In fact, from table 6 of present application, it can be seen that the *Photinus pyralis* luciferase mutant D357K (identified as Enzyme No. 25) is not thermostable (0.1% activity remaining after incubation at 45° for 4 minutes, as compared to 0.05% activity of the wild-type enzyme tested at the same conditions). Moreover, in table 4 of present application, it can be seen that the same mutant D357K shows a deviation from wild-type luciferase of only 2nm, in terms of wavelength of emitted light. These differences are considered not to be high enough to distinguish the scope of said mutant from the one of the wild-type luciferase.

- 6.2). Having regard to the above comments, it should be concluded that the residues of the luciferases, as indicated in claim 1 cannot always be defined unambiguously and, as such, claim 1 fails to comply with the requirements of Article 6 PCT with respect to clarity.

Moreover, the identification of residues in any luciferase (wild-type ones and the ones having at least 60% similarity to said wild-type enzyme), corresponding to the one of *Photinus pyralis* luciferase mentioned, and whose mutation would lead to a luciferase with enhanced thermostability and/or able to emit light at a different wavelength as compared to the corresponding wild-type luciferase, would put an undue burden to the person skilled in the art.

Consequently, present application does not meet the requirements of Articles 5 and 6 PCT because the subject-matter of claim 1 is not sufficiently disclosed and supported over its whole breadth.

- 7). In amended claim 11(h), residue 108 of *Luciola lateralis* luciferase is indicated as being corresponding to residue 105 of *Photinus pyralis* luciferase. However, from the alignment shown in figure 2 of document D2, it seems that the *Luciola lateralis* luciferase residue corresponding to *Photinus pyralis* luciferase Alanine residue 105 is the Threonine residue in position 107, instead (Article 6 PCT).
Same remark applies also to the passage on amended page 9, lines 35-36, on amended page 12, line 6, and on amended page 16, lines 7-8.
- 8). In page 5 of present application it is cited that in the luciferase Ph_{RE} of *Phrixothrix*, the residue corresponding to residue 357 of *Photinus pyralis* luciferase is the residue Leucine in position 354. Having regard to the sequence alignment in figure 5 of document D3, it seems that the corresponding residue is the Leucine residue on position 355 of *Phrixothrix* Ph_{RE} luciferase, instead (Article 6 PCT).
- 9). Claim 7 does not meet the requirements of Article 6 PCT because the amino acids indicated as uncharged polar are not explicitly defined and there is no general consensus in the technical field about the amino acids belonging to this class. In fact, document D4, provided by the Applicant during the procedure, indicates on page 57 that amino acid residues with uncharged polar side chains are Asparagine, Glutamine, Serine, Threonine and Tyrosine. Document D5, however, shows that neutral polar amino acid residues are Serine, Threonine, Tyrosine, Tryptophan, Asparagine, Glutamine and Cysteine. Moreover, in the description on page 5, present application refers to uncharged polar amino acids such as Tyrosine, Asparagine, Glutamine, Phenylalanine, Serine, Tryptophan or Threonine.